Original Article

Role of Endothelin-1/Endothelin Receptor System in Endotoxic Shock Rats

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Endothelin (ET)-1, a potent vasoconstrictor peptide derived from the endothelium, is markedly increased in endotoxic shock, although the pathophysiological role of ET-1 under septic conditions remains obscure. To delineate the role of ET-1 and its receptor subtype in endotoxic shock, we here attempted to determine the changes of circulating levels of ET-1 and its biosynthetic intermediate big ET-1 in endotoxic shock rats, to evaluate the gene expression of ET-1 as well as the ET-1 receptor subtypes (ET_A and ET_B) in the heart, lung and liver, and to study the effects of ET receptor antagonists on systemic arterial blood pressure, heart rate and survival rate. Administration of bacterial lipopolysaccharide (LPS) caused profound hypotension, increased heart rate and death, and these effects were blocked by a nonselective ET_A/ET_B receptor antagonist (TAK044), but not by an ET_A selective antagonist (BQ123). Administration of exogenous ET-1 caused a profound pressor response in control rats, but not in the LPS-pretreated rats. Injection of LPS caused marked elevation of plasma levels of both ET-1 and big ET-1, which were not affected by treatment with either ET receptor antagonist. Administration of LPS caused up-regulation of ET-1 and ET_B receptor mRNA in the heart, whereas ET_A receptor mRNA was markedly down-regulated in the heart, lung and liver. These data suggest differential gene regulation of ET-1 and its receptor subtypes in various organs from endotoxic shock rats, and that nonselective ET_A/ET_B receptor antagonist, but not ET_A receptor antagonist, ameliorates endotoxin-induced hypotension and death. (*Hypertens Res* 2001; 24: 119-126)

Key Words: endotoxin shock, endothelin-1, ET receptor antagonist, gene expression, real-time quantitative PCR

Introduction

Endothelin (ET)-1 is a potent endothelium-derived vasoconstrictor peptide (1). Circulating ET-1 increases in several cardiovascular diseases including endotoxic shock (2, 3). It has been reported that serum concentrations of ET-1 were markedly increased not only in experimental endotoxic shock models, such as rats (4), dogs (5, 6), pigs (7), and sheep (8), but also in human subjects with sepsis and septic shock (9, 10). It has been suggested that exces-

sive release of ET-1 during sepsis may serve to maintain blood pressure, whereas increased circulating ET-1 under septic conditions may contribute to the reduction of regional blood flow in vital organ beds (3). Therefore, it remains uncertain whether endogenous ET-1 may contribute to the underlying pathophysiology of multiple organ failure associated with endotoxic shock or to the compensatory response to the endotoxic shock.

Two distinct subtypes of ET receptor have been identified thus far, namely, an ET-1-selective ET_A receptor and a non-ET isopeptide-selective ET_B receptor (2). ET_A

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receptor in vascular smooth muscle cells mediates vasoconstriction, while ET_B receptor in endothelial cells mediates vasodilation by releasing nitric oxide (NO) and prostacyclin from the endothelium (11, 12). However, it has recently been reported that ET_B receptor located on certain vascular smooth muscles also mediates vasoconstriction (13, 14).

Recently, several ET receptor antagonists have been developed and used for the treatment of various diseases (15, 16). A selective ET_A receptor antagonist, BQ123, has been shown to attenuate ET-1-induced contraction of the pulmonary artery in endotoxemic rats (17), suggesting the role of ET_A receptor activation in the development of pulmonary vasoconstriction during sepsis. On the other hand, a nonselective ET_A/ET_B receptor antagonist, bosentan, has been shown to abolish pulmonary hypertension, improve cardiac output and increase regional blood flow in porcine endotoxemic shock (18, 19), suggesting the importance of both ET_A and ET_B receptors in the maintenance of blood pressure.

These observations led us 1) to study the effects of antagonists for selective ET_A receptor and nonselective ET_A/ET_B receptor on systemic hemodynamics and survival in a rat model of endotoxemic shock, 2) to measure the changes of circulating concentrations of ET-1 and big ET-1, and 3) to evaluate gene expressions of ET-1 and ET receptor subtypes in major organs in the endotoxemic shock model.

Methods

Reagents

LPS was purchased from Sigma (St. Louis, USA). TAK044 was supplied by Takeda Chemical Industry (Osaka, Japan) and BQ123 by Banyu Pharmaceutical Co. (Tsukuba, Japan). Pentobarbital sodium (Nembutal®) was purchased from Dainabot (Osaka, Japan).

Measurement of Hemodynamic Changes

The experimental procedures described here were in accordance with the institutional guidelines for animal studies. Male Wistar rats (300–350 g, Sankyo, Japan) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Anesthesia was maintained by supplementary injections of pentobarbital sodium (approximately 5–10 mg/kg, i.p., as required). The femoral vein was cannulated for the administration of drugs, and the femoral artery for continuous recordings of phasic pressure, mean arterial pressure (MAP), and heart rate (HR) using a pressure transducer (Nihon Kohden, Tokyo, Japan). These parameters were continuously displayed on a polygraph. After a 30-min equilibrium period, animals received intravenous administration of BQ123 (30 mg/kg body weight),

TAK044 (1 mg/kg) or vehicle (saline) as a bolus and, 30 min later, a 10-min continuous infusion of LPS (20 mg/kg) or vehicle (saline). The dose (1 mg/kg) of TAK044 was selected from a previous study (20), where it completely blocked both ET_A receptor-mediated vasopressor and ET_B receptor-mediated vasodepressor responses by ET-1 in rats. Five hours after injection of LPS or vehicle, blood was withdrawn and animals were sacrificed. The heart, lung, and liver were removed and immediately frozen in liquid nitrogen. In rats dying earlier than 5 h, blood sampling was performed shortly before death, and tissue samples were removed immediately after death. Collected blood samples were mixed with EDTA and plasma was separated by centrifugation. Tissue and plasma samples were stored at –80°C until analysis. To determine the functional significance of changes of ET receptor subtype expression in endotoxemia, exogenous ET-1 (0.1 nmol/kg) was administered 2 h after injection of LPS (20 mg/kg), followed by MAP measurement for 30 min.

Measurement of Plasma ET-1 and Big ET-1

Plasma samples were extracted using Sep-Pak C-18 columns as reported previously (21). Recoveries of both ET-1 and big ET-1 were 50–60%. ET-1 was determined by RIA using an ET-1 assay kit (Amersham Pharmacia Biotech Inc., Piscataway, USA); the sensitivity was 0.2 fmol/tube and the cross-reactivity for ET-1, ET-2, ET-3 and big ET-1 are 100, 144, 52 and 0.26%, respectively. Big ET-1 concentration was determined by ELISA using a big ET-1 assay kit (Immuno-Biological Lab., Fujioka, Japan); the sensitivity was 0.18 fmol/ml with no cross-reactions with big ET-3 or ET-1. All samples were assayed in duplicate.

Quantification of ET-1, ET_A Receptor and ET_B Receptor mRNA by a Real-Time Quantitative RT-PCR

Total RNA was extracted from tissue specimens (50 mg) homogenized in 1 ml RNAzol B™ (TEL-TEST, Inc., Friendswood, USA). cDNA was synthesized from RNA (1.2 mg) with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Inc.) using the random primer, pd(N)₆. Real-time quantitative PCR was performed using a LightCycler™ (Roche Boehringer, Mannheim, Germany) (22, 23). The sequences of primers for rat ET-1, ET_A receptor and ET_B receptor were as follows: ET-1: forward primer 5'-CGTCCCGTATGGACTAGGAA-3' and reverse primer 5'-TGCAACTCGAAAGGAGGTCT-3', ET_A receptor: forward primer 5'-ATCGGGATCCCC TTGATTAC-3' and reverse primer 5'-TGACAACCAAGCAGAAGACG-3', and ET_B receptor: forward primer 5'-GGACTACAAAGGAAAGCCCC-3' and reverse primer 5'-TGCTCACCAATACAGAGCG-3'. One micro-

Fig. 1. Effects of ET receptor antagonists on mean arterial pressure (MAP) in endotoxic shock rats. Anesthetized rats for each group received (i) vehicle (saline) only as a control ($\square$, $n=6$), (ii) LPS (20 mg/kg) alone ($\blacksquare$, $n=9$), (iii) BQ123 (30 mg/kg) alone ($\circ$, $n=4$), (iv) BQ123+LPS ($\bullet$, $n=4$), (v) TAK044 (1 mg/kg) alone ($\triangle$, $n=4$), or (vi) TAK044+LPS ($\blacktriangle$, $n=6$). * $p<0.05$ and ** $p<0.01$ vs. control at the same time point.

Fig. 2. Effects of ET receptor antagonists on heart rate in endotoxic shock rats. Anesthetized rats for each group received (i) vehicle (saline) only as a control ($\square$, $n=6$), (ii) LPS (20 mg/kg) alone ($\blacksquare$, $n=9$), (iii) BQ123 (30 mg/kg) alone ($\circ$, $n=4$), (iv) BQ123+LPS ($\bullet$, $n=4$), (v) TAK044 (1 mg/kg) alone ($\triangle$, $n=4$), or (vi) TAK044+LPS ($\blacktriangle$, $n=6$). * $p<0.05$ and ** $p<0.01$ vs. control at the same time point.

liter cDNA template was mixed with 9 μ l reaction mixture containing 4 μ M $MgCl_2$ and 0.5 μ M primers, placed in a reaction capillary and amplified. Temperature profiles for amplification were optimized for each set of primers: 95°C for 1 min followed by 40 cycles of 95°C for 1 s, 60°C for 5 s, 72°C for 20 s, except that the annealing temperatures for ET_A and ET_B receptors were 57°C and 54°C, respectively. The temperature ramp rate was 20°C. Following the completion of each extension step at 72°C, the fluorescence of each sample was measured at 82°C to exclude any possible non-specific count. After the completion of amplification, the products were subjected to a temperature gradient from 65°C to 95°C at 0.2°C/s with continuous fluorescence monitoring to produce a melting profile of the products. The fluorescence data were quantitatively analyzed using standard controls of known concentration to produce a standard curve, allowing quantification of templates.

Statistical Evaluation

All values are expressed as the mean $\pm$ SEM. Data were statistically analyzed with one-way analysis of variance (ANOVA) using StatView 5.0 software for Macintosh.

Results

Effects of ET Receptor Antagonists on Systemic Hemodynamics and Death in Endotoxic Shock Rats

Baseline values of MAP and HR were not significantly different among experimental groups. Injection of LPS caused a marked fall in MAP (from 91.2 ± 5.7 mmHg to 48.5 ± 3.2 mmHg, $p<0.01$) after 5 h (Fig. 1). In the BQ123-pretreated group, administration of LPS caused an earlier fall in MAP with a maximal fall to 58.0 ± 7.2 mmHg ($p<0.01$) after 4 h. In contrast, pretreatment with TAK044 prevented LPS-induced fall in MAP after 5 h; there was no significant difference in MAP between the TAK044+LPS group and the control group.

Throughout the observation period, HR increased persistently in all groups (Fig. 2). The increase in HR was greater in the LPS group (562.2 ± 11.9 beats/min at 4 h) and the BQ123+LPS group (609.7 ± 17.4 beats/min at 4 h) than in the control group, while the HR of the TAK044+LPS group did not differ from that of the control group.

In the LPS group, the mortality rate was 33.3% at 5 h (Fig. 3). TAK044 saved the rats from LPS-caused death and all rats survived after 5 h. In contrast, BQ123 increased the mortality rate (25%) at 3 h and all rats died before 4 h.

Injection of ET-1 (0.1 nmol/kg) into control rats caused a profound pressor response (19.3 ± 2.6 mmHg), whereas ET-1 failed to induce a pressor response in LPS-pre-

Fig. 3. Effects of ET receptor antagonists on survival rate in endotoxic rats. Anesthetized rats for each group received (i) vehicle (saline) only as a control (□, n=6), (ii) LPS (20 mg/kg) alone (■, n=9), (iii) BQ123+LPS (●, n=4), or (iv) TAK044+LPS (▲, n=6).

Fig. 4. Effect of exogenous ET-1 on MAP in normal and endotoxic shock rats. ET-1 (0.1 nmol/kg) was administered to anesthetized rats 2 h after treatment with or without LPS (20 mg/kg) and MAP was recorded. LPS (■, n=7), ET-1 (▲, n=4), LPS+ET-1 (●, n=3).

Fig. 5. Plasma ET-1 and big ET-1 levels in endotoxic shock rats. Anesthetized rats for each group received (i) saline as a control (□, n=6), (ii) LPS (20 mg/kg) (■, n=9), (iii) BQ123 (30 mg/kg)+LPS (▨, n=4), or (iv) TAK044 (1 mg/kg)+LPS (▩, n=6). * $p<0.05$ and ** $p<0.01$ vs. control.

treated rats (Fig. 4).

Changes of Circulating ET-1 and Big ET-1 in Endotoxic Shock Rats

After LPS administration, plasma concentrations of ET-1 (9.6 ± 0.8 pg/ml) and big ET-1 (61.8 ± 13.8 pg/ml) were significantly ($p<0.01$) increased compared to those of controls (2.7 ± 0.34 pg/ml, 18.2 ± 3.9 pg/ml), and these changes were unaffected by pretreatment with BQ123 or TAK044 (Fig. 5).

Changes of ET-1, ET_A and ET_B Receptor mRNA Expression in Major Organs of Endotoxic Shock Rats

Steady-state mRNA levels of both ET-1 and ET_B receptor in the heart significantly ($p<0.05$) increased ($220.3 \pm 17.3\%$, $199.2 \pm 21.6\%$, respectively) after the administration of LPS compared to those of vehicle (Fig. 6). In contrast, steady-state ET_A receptor mRNA levels in the heart were markedly decreased ($33.1 \pm 1.9\%$, $p<0.01$) after LPS administration compared to vehicle. None of ET-1, ET_A or ET_B mRNA expression after LPS administration was affected by pretreatment with BQ123 or TAK044.

Fig. 6. Changes of relative mRNA levels of ET-1, ET_A and ET_B receptor in the heart of endotoxic shock rats. Data are obtained and plotted for the same group and in the same manner as in Fig. 3. mRNA expression was quantified by a real-time quantitative RT-PCR. * $p < 0.05$ and ** $p < 0.01$ vs. control.

Fig. 7. Changes of relative mRNA levels of ET-1, ET_A and ET_B receptor in the lung of endotoxic shock rats. Data are obtained and plotted for the same group and in the same manner as in Fig. 3. * $p < 0.05$ vs. control.

In the lung, however, neither ET-1 nor ET_B receptor mRNA levels changed, whereas the ET_A receptor mRNA level was significantly ($p < 0.05$) reduced ($56.3 \pm 8.7\%$) after LPS administration (Fig. 7). These changes were unaffected by pretreatment with BQ123 or TAK044.

In the liver, administration of LPS significantly ($p < 0.05$) increased ET-1 mRNA levels ($203.8 \pm 32.4\%$), but markedly decreased the ET_A receptor ($15.1 \pm 5.8\%$, $p < 0.01$), while ET_B receptor mRNA levels did not change (Fig. 8); these changes were unaffected by pretreatment

with BQ123 or TAK044.

Discussion

In the present study, administration of bacterial endotoxin to anesthetized rats caused a profound hypotension and increase in heart rate associated with marked increases in plasma ET-1 levels. These data are consistent with those of previous studies in septic shock patients (9, 10) as well as in several experimental animal models (4, 8). It has

Fig. 8. Changes of relative mRNA levels of ET-1, ET_A and ET_B receptor in the liver of endotoxic shock rats. Data are obtained and plotted for the same group and in the same manner as in Fig. 3. * $p < 0.05$ and ** $p < 0.01$ vs. control.

been shown that secretion of ET-1 from endothelial cells is stimulated by many factors, such as bacterial endotoxin, cytokines, angiotensin, and vasopressin (2, 4, 24, 25). Therefore, it is most likely that the increase in plasma ET-1 in endotoxic shock is mainly derived from the endothelium stimulated by these multiple factors known to be increased under endotoxemia. Furthermore, the present finding of increased plasma levels of big ET-1, a biosynthetic intermediate, suggests that synthesis and/or processing of ET-1 is also enhanced in endotoxemia. These data are consistent with those of a previous study using a porcine endotoxic shock model (18).

The present study using a quantitative RT-PCR method clearly revealed that steady-state ET-1 mRNA levels were significantly enhanced in the heart and the liver, but not in the lung, of endotoxic shock rats. Several previous studies using Northern blot analysis have shown that the expression of ET-1 mRNA is increased in various major organs, including the aorta and lung, of endotoxic shock animal models (18, 26-28). The reason for the lack of an increase in ET-1 mRNA in the lung in this study is not clear, but may be explained by the timing of organ removal and/or the methods of mRNA quantification employed.

Despite the extensive studies thus far reported on enhanced ET-1 expression and secretion in endotoxic shock, there has been no detailed information as to the changes of gene expression of ET receptor subtype in the major organs affected in these animal models. The present study using a sensitive RT-PCR method demonstrated for the first time that ET receptor subtype genes were differentially regulated in major organs in endotoxic shock rats. Namely, ET_A receptor mRNA levels were markedly

decreased in the heart, lung and liver of endotoxic shock rats, and these changes were not affected by coadministration of BQ123 or TAK 044. The reason for the marked down-regulation of ET_A receptors in these organs remains unknown, but several possibilities can be raised. The expression of ET_A receptors may be conversely decreased by increasing circulating ET-1. Increased circulating cytokines, growth factors and glucocorticoid during sepsis may also be responsible. In fact, it has been reported that ET_A receptors are down-regulated by platelet-derived growth factor, transforming growth factor β (2) and glucocorticoid (29).

In contrast, the present study showed that ET_B receptor mRNA levels increased in the heart, but remained unchanged in the lung and liver of endotoxic shock rats. The molecular mechanism by which the ET_B receptor gene is up-regulated only in the myocardium from endotoxic shock rats remains unknown. Since the expression of ET_B receptor is increased by cyclic AMP (30) and angiotensin II (31), increased secretion of catecholamines and angiotensin II during sepsis may be responsible for the up-regulation of cardiac ET_B receptor. Whatever the mechanisms, it remains to be determined whether the differential gene expression of the ET receptor subtype is regulated by transcriptional and/or posttranscriptional level. In contrast to the profound pressor response of exogenous ET-1 in control rats, administration of exogenous ET-1 failed to increase MAP in the LPS-pretreated rats. These results could be interpreted as a functional consequence of the observed changes of the expression of ET receptor subtypes. Down-regulation of ET_A receptors may reduce ET_A receptor-mediated vasoconstrictor response, whereas up-regulation of ET_B receptors may en-

hance ET_B receptor-mediated vasodilator response.

Recently, several ET receptor antagonists have been developed (16)—including BQ123, a selective ET_A receptor antagonist, and TAK044, a nonselective ET_A/ET_B receptor antagonist—and used in several experimental models (6, 15). We have therefore attempted to delineate the role of ET-1 and ET receptor subtype in rat endotoxic shock models using these ET receptor antagonists. The present study showed that administration of BQ123, but not TAK044, aggravated the LPS-induced decrease in MAP accompanied by a baroreflex-mediated increase in HR. These data are comparable with those of a previous study using an ET receptor antagonist (SB209670) that augmented the hypotension and vascular hyporeactivity to noradrenaline in the endotoxic shock models (32). Taken together, these results suggested that ET_A receptor is mainly responsible for the maintenance of blood pressure in the rat model of endotoxic shock. In contrast, recent studies have demonstrated the beneficial effects of ET_A receptor antagonists in rat and canine models of chronic heart failure (33, 34), in which both the ET-1 and ET_A receptor were shown to be markedly up-regulated in the myocardium. ET-1 has been shown to induce hypertrophic responses of cardiomyocytes *via* ET_A receptor (35). Furthermore, it has been shown that ET-1 ameliorates the LPS-induced myocardial contractile dysfunction *via* ET_A receptor-mediated blockade of NO production (36). Thus, it is possible to speculate that chronic treatment with ET_A receptor antagonists may alleviate ET_A-mediated cardiac remodeling as well as NO-mediated impaired myocardial contractility in the failing heart.

In the present study, a nonselective ET_A/ET_B receptor antagonist (TAK044), but not an ET_A selective antagonist (BQ123), increased the survival rate and improved the blood pressure of endotoxic shock rats. These observations are consistent with those on a porcine endotoxic shock model, in which a nonselective ET_A/ET_B receptor antagonist, bosentan, improved survival rate (18). Further, it was shown that bosentan can abolish pulmonary hypertension, improve cardiac output, and increase regional blood flow without a further decrease in MAP (18). Taken together, these results suggest that nonselective ET_A/ET_B receptor antagonists may well ameliorate the LPS-induced hypotension and subsequent death in rats. However, it remains to be determined whether a selective blockade of ET_B receptor is more beneficial than a nonselective blockade of both ET_A and ET_B receptors in endotoxic shock rats, because it has been reported that administration of a selective ET_B receptor antagonist, RES-701-1, caused adverse hemodynamic effects in congestive heart failure dogs (34).

ET-1 has recently been shown to play protective roles against apoptotic cell death in various cell types, including cardiovascular tissue cells. For example, ET-1 protects endothelial cells and cardiomyocytes from apoptosis in-

duced by growth factor deprivation, NO and natriuretic peptides *via* ET_B receptor (37, 38) and protects vascular smooth muscle cells *via* ET_A receptor (39). Therefore, concomitant up-regulation of ET-1 and the ET_B receptor in endotoxemia may act as an adaptive mechanism to prevent apoptotic elimination of the injured endothelium and myocardium.

In conclusion, the present study showed that ET-1 and the ET_B receptor genes are up-regulated in the heart, whereas the ET_A receptor gene is down-regulated in the heart, lung, and liver of endotoxic shock rats, suggesting differential gene regulation of the ET-1/ET receptor subtype system in endotoxemia. Furthermore, the nonselective ET_A/ET_B receptor antagonist, but not the ET_A selective antagonist, ameliorates endotoxin-induced hypotension and death. Therefore, ET_A receptor may play a compensatory vasoconstrictor role for maintenance of blood pressure in endotoxic shock rats, whereas the pathophysiological role of ET_B receptor in endotoxemia remains open to question.

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